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Mutations in Cognate Genes of *Saccharomyces cerevisiae hsp70* Result in Reduced Growth Rates at Low Temperatures

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Expression of two *Saccharomyces cerevisiae* genes (*YG101* and *YG103*) that are related to the gene encoding inducible 70K protein (*hsp70*) is repressed upon heat shock. Mutations of the two genes were constructed in vitro and substituted into the yeast genome in place of the wild-type alleles. No phenotypic effect of single mutations of either gene was detected. However, cells containing both *YG101* and *YG103* mutations showed altered growth properties; double-mutation cells possess an optimal growth temperature of 37°C rather than 30°C and grow increasingly poorly as the temperature is lowered. Mutations of two other members of this *hsp70*-related multigene family, *YG100* and *YG102*, have been analyzed (E. A. Craig and K. Jacobsen, Cell 38:841–849, 1984). Cells containing both *YG100* and *YG102* mutations cannot form colonies at 37°C. Fusions between the *YG101* and *YG102* promoter regions and the *YG100* and *YG101* structural genes, respectively, were constructed. The *YG101* promoter-*YG100* structural gene fusion was not able to restore normal growth properties to the *yg101*$^-$ *yg103*$^-$ mutant. Also, *yg100*$^-$ *yg102*$^-$ cells containing the *YG102* promoter-*YG101* structural gene fusion were unable to grow at 37°C. Failure of the protein products of related genes to rescue the relative cold sensitivity of growth suggests that members of the *hsp70* multigene family are functionally distinct.

The genomes of a wide variety of plant, animal, and bacterial species contain genes related to the major heat-inducible gene (*hsp70*) of *Drosophila melanogaster*, the organism in which the heat shock response has been most thoroughly studied (2). The procaryote *Escherichia coli* has a single related gene, *dnaK* (3), which is expressed abundantly under all growth conditions (12). DnaK is about the seventh most abundant protein by weight fraction during growth at 30°C. Furthermore, DnaK is a heat shock protein, its rate of synthesis being rapidly and transiently enhanced upon temperature upshift. Eucaryotes have evolved families of related genes, some of which are expressed only after a temperature upshift, while others are expressed under normal steady-state growth conditions. Multigene families have been identified in yeast, fruit fly, mouse, and human cells (5, 13, 14, 21, 36).

Eight genes related to the *Drosophila hsp70* genes have been identified in the *Saccharomyces cerevisiae* genome and isolated as recombinant DNA clones (7). The gene sequence relationships among the family members, designated *YG100* through *YG107*, is complex, with homologies ranging from 45 to 96% identity of nucleotide sequence. Expression of family members is modulated differently in response to temperature shifts and during steady-state growth at various temperatures. For example, expression of two genes (*YG106* and *YG107*) was not detected during steady-state growth at 23°C, while their expression is greatly enhanced upon upshift to 38°C (7). Expression of some of the other family members changes little if at all upon heat shock. Two members of the family, *YG100* and *YG102*, have been studied in the greatest detail. These two genes are 96% identical in the protein coding region at the nucleotide level, but are regulated differently. *YG102* is expressed at a high level during steady-state growth, and the expression changes little upon a temperature upshift. *YG100* expression levels are severalfold lower than those of *YG102* during steady-state growth, but its transcription is enhanced during a heat shock (9). Strains containing mutations in either *YG100* or *YG102* show no obvious phenotype, but strains lacking both wild-type *YG100* and *YG102* genes have dramatically altered growth properties. Although growth is slower at all temperatures, the severity of the effect increases with increasing temperatures; the double-mutation strain is unable to form colonies at 37°C (6). Here we report the construction and analysis of mutations in two other closely related members of the family, *YG101* and *YG103*. Unlike other members of the family, expression of these two genes is repressed upon a temperature upshift.

The function of the *hsp70*-related genes as well as other heat shock genes remains obscure. Although it is known that DnaK is required for replication of phage λ (11, 30), its role in the metabolism of the host is not clear. In eucaryotes, the question arises as to whether the related proteins perform the same or distinct functions. We seek here to determine whether a protein whose expression is decreased upon a temperature upshift is functionally distinct from a protein whose expression is enhanced.

MATERIALS AND METHODS

Strains, culture conditions, and transformation. The *S. cerevisiae* strains used in this study are listed in Table 1. Transformations were carried out using LiCl as previously described (6). The yeast culture media used in this study have been described previously (6, 31).

Hybridization analysis. DNA was isolated from yeast cells as described (6, 31) and digested with restriction enzymes according to the instructions provided by the commercial supplier. The digested DNA was ethanol precipitated, and DNA fragments were separated in horizontal 1% agarose gels. The DNA was transferred to nitrocellulose paper (Schleicher & Schuell, Inc.) by the method of Southern (33). The immobilized DNA was hybridized to probe labeled with [^{32}P]dCTP (Amersham Corp.) by nick translation as previously described (6). The hybridization was performed at

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TABLE 1. Strains used in this study[a]

Strain	Genotype
X46	a *ade2-101 lys2 ura3-52 Δtrp1 leu2-3,112* / α + + *ura3-52 Δtrp1 leu2-3,112 his4-713*
T18	α *his4-713 leu2-3 trp1-7*
T88	α *leu2-3,112 his3-11,15 can1*
X46-8	a *ade2-101 lys2 ura3-52 Δtrp1 leu2-3,112* [*YG101*H1:*LEU2*(G)]
T145	α *leu2-3,112 his3-11,15* [*YG103*CB:*HIS3*(G)]
X81-5D	a *ura3-52 Δtrp1 his3-11,15 leu2-3,112* [*YG101*H1:*LEU2*(G)]
X89I	a *ura3-52 his3-11,15 Δtrp leu2-3,112* / α + *his3-11,15* + *leu2-3,112* [YG101H1:LEU2(G)] [YG103CB:HIS3(G)]
T134	α *ade2-10 ura3-52 Δtrp1 leu2-3,112* [*YG101*H1:*LEU2*(G)]
T128	α *ade2-10 his4-713 Δtrp1 lys2* [*YG102:LEU²*(G),*YG100:LEU²*(G)]

[a] X46 and T128 were described previously (6). X46 and T88 were transformation recipients for the *YG101* and *YG103* mutations, respectively. Sporulation of the X46 transformant resulted in haploids containing the *YG101* mutation. One of these haploids, X46-8, was used in much of the subsequent analysis. One of the *YG103* transformants, T145, was used for much of the subsequent analysis. To construct a strain containing both a *YG101* mutation and a *HIS3* mutation, T88 and X46-8 were mated, and the diploid was sporulated to obtain the haploid strain X81-5D. X81-5D and T145 were mated to obtain a diploid (X89I) heterozygous for the *YG101* and *YG103* mutations.

37°C in 50% formamide–0.75 M NaCl–0.075 M sodium citrate–0.2% sodium dodecyl sulfate (SDS)–10 mM EDTA–1× Denhardt solution (8). The filters were washed at 65°C sequentially in 5× SSC–0.2% SDS, 1× SSC–0.2% SDS, and 0.1× SSC–0.2% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

RNA was extracted as previously described (14) and electrophoresed on 1% agarose gels containing 1% formaldehyde, 20 mM sodium morpholinepropanesulfonic acid (pH 7), 5 mM sodium acetate, and 1 mM EDTA. The gel was incubated in 20× SSC at room temperature for 1 h, and the RNA was then blotted onto nitrocellulose. Hybridization was carried out at 49°C in 50% formamide–0.15 M NaCl–0.15 M Tris hydrochloride (pH 7.0)–1 mM EDTA–0.2% SDS–2× Denhardt solution–10% dextran sulfate–calf thymus DNA (2 mg/ml). To eliminate cross-hybridization with less homologous members of the family, the final wash was carried out in low salt. The washing scheme carried out in 0.2% NaPPi–0.1% SDS–25 M $NaPO_4$ (pH 7.0) and decreasing concentrations of SET (5× SET is 0.75 M NaCl plus 0.15 M Tris hydrochloride [pH 8] plus 10 M EDTA) was as follows: 5 min at room temperature in 5× SET, 30 min at 68°C in 1× SET, followed by 2× SET and then 0.05× SET.

Plasmid construction. The starting materials for the construction of the *YG101* and *YG103* mutations were the clone YG101H1, a 3.7-kilobase (kb) *Hin*dIII fragment inserted into pBR322 (14), and YG103CB, a 3.8-kb *Cla*I-*Bam*HI fragment inserted into pBR322, containing *YG101* and *YG103* genes, respectively. B2 and E3 are clones isolated from a library (constructed by M. Mendenhall) of S288C yeast DNA, partially digested with *Sau*3A and inserted into the *Bam*HI site of YIp5. B2, containing the entire *YG101* gene, and E3, containing the entire *YG103* gene, contain inserts of 12 and 11 kb, respectively. YIp5 contains the yeast *URA3* gene, which complements the *ura3-52* mutation.

Both the *YG102* promoter-*YG101* structural gene and the *YG101* promoter-*YG100* structural gene fusions were constructed in the centromere containing vector YCp50, which also contains the *URA3* gene, *CEN4*, and *ARS1* (15). The fusion between the *YG102* promoter and the *YG101* structural gene was formed by ligation of two *Sau*3A ends located at position −31 in the *YG102* gene and position −26 in the *YG101* gene (+1 being the A of the initiation codon). Since the sites of the 5′ ends of the mRNAs occur at positions −33 through −50 and at −45, respectively, of *YG102* and *YG101*, the fusion occurs within the 5′ transcribed but untranslated region. The fusion between the *YG101* promoter and the *YG100* gene was made via ligation of *Hin*dIII ends. The *YG101* gene has a *Sau*3A site at −26. The *Sau*3A site was cloned into the *Bgl*II site of a polylinker, placing it adjacent to the *Xba*I and *Hin*dIII sites. A *YG100* gene with a *Hin*dIII site at position −16 was constructed by D. Stone by digesting *YG100* with *Bal*31 and adding *Hin*dIII linkers. Since the 5′ ends of the transcripts of *YG101* and *YG100* are at −45 and −55, respectively, the fusion site is in the 5′ transcribed but untranslated region. The *102-101* and *101-100* fusions contain approximately 755 and 650 bases of 5′ upstream regions, respectively; fragments of this size have been shown to be able to drive regulated transcription when fused to the *E. coli lacZ* gene (9).

Protein labeling. Cells (3×10^6) grown in supplemented minimal medium lacking methionine and uracil at 23°C were labeled with [^{35}S]methionine (33 μCi/ml). The cells were pelleted, frozen at −70°C, and then prepared for electrophoresis by the procedure of Miller et al. (24), with some modifications. The pellets were resuspended in 200 μl of 20 mM Tris hydrochloride (pH 8.8)–2 mM $CaCl_2$ and mixed in a vortex mixer in the presence of 0.17-g glass beads. The lysate was treated for 5 min at 4°C with 10 μl of micrococcal nuclease (1.0 mg/ml in Tris hydrochloride-$CaCl_2$). Twenty microliters of 2% SDS–10% β-mercaptoethanol was added, followed by 20 μl of pancreatic DNase I (1.0 mg/ml) and 1 μl of sonication buffer (0.01 M Tris hydrochloride [pH 7.4], 1 mM $MgCl_2$, pancreatic RNase [50 μg/ml]), and incubation was continued at 4°C for 5 min. The samples were lyophilized and resuspended in 100 μl of O'Farrell's buffer A (26). Electrophoresis was carried out essentially by the method of O'Farrell (26).

RESULTS

Expression of *YG101* and *YG103* is repressed upon heat shock. We examined the expression of the *YG101* and *YG103* genes by analyzing the levels of transcripts of these genes under a number of temperature conditions (Fig. 1). Cellular RNAs that had been separated on denaturing gels and blotted to nitrocellulose filters were hybridized with a labeled *YG101* clone under conditions that allowed cross-hybridization with the highly homologous *YG103* transcripts, but not with the other family members (7). The levels of transcripts of the two genes (which are indistinguishable in size in our electrophoresis conditions) decreased as the temperature of steady-state growth was increased from 23 to 39°C. Thirty minutes after an upshift from 23 to 39°C the levels dropped dramatically from their preshift levels. Shifts to intermediate temperatures resulted in a more modest drop in RNA levels.

Construction and analysis of *YG101* and *YG103* mutations.

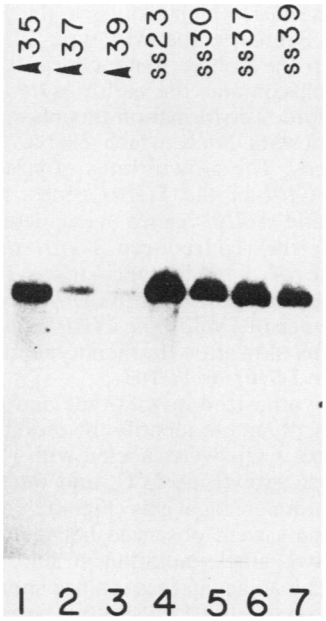


FIG. 1. Expression during steady-state growth and heat shock. RNA extracted from cells growing logarithmically at 23°C (lane 4), 30°C (lane 5), 37°C (lane 6), and 39°C (lane 7) and from cells 30 min after a shift from 23 to 35°C (lane 1), 37°C (lane 2), and 39°C (lane 3). Portions (3 μg) of the RNA preparations were electrophoresed on a 1% formaldehyde agarose and then blotted to nitrocellulose. A probe labeled with ^{32}P was prepared by nick translation of the plasmid YG101H1 which contains the entire protein coding region of *YG101* (14).

Mutations in *YG101* and *YG103* were constructed in vitro. We then utilized the one-step gene replacement method of Rothstein (28) to integrate the mutations into the yeast genome and simultaneously delete the wild-type gene. Our strategy for constructing the mutations is diagrammed in Fig. 2. Plasmids containing *YG101* and *YG103* were digested with *Bgl*II, which cleaves at the nucleotides encoding *aa328*, and ligated with a 3-kb *Bgl*II fragment carrying the *S. cerevisiae LEU2* gene or a 1.7-kb *Bam*HI fragment carrying the *S. cerevisiae HIS3* gene. The resulting plasmids, YG101H1:LEU2(G) and YG103CB:HIS3(G), were cleaved with *Hin*dIII and *Cla*I-*Bam*HI, respectively, to separate yeast from vector sequences. The digested DNAs were used to transform diploids since it was not known whether a *YG101* or *YG103* mutation would result in lethality in the absence of a wild-type gene. To identify those transformants with the desired molecular structure, genomic DNA was prepared and analyzed by the method of Southern (33). Most transformants gave hybridization patterns consistent with the simple replacement of the wild-type gene with the mutant gene in one chromosomal homolog.

To determine whether haploid strains containing no wild-type *YG101* or *YG103* gene show an altered phenotype, haploid meiotic segregants were derived from the transformants. A high percentage of the resulting spores were viable, and all four spore clones from tetrads displayed a normal Mendelian (2:2) segregation of Leu^+:Leu^- in the case of *YG101* and His^+:His^- in the case of *YG103*. We verified by hybridization analysis that the Leu^+ and His^+ cells contained the expected mutant genes but not the corresponding wild-type gene. Under conditions of high stringency, two fragments (4.0 and 2.1 kb) representing *YG101* and *YG103*, respectively, hybridized to the *Cla*I-*Hin*dIII fragment from the protein coding region of *YG101* used as a probe (Fig. 2c). In a single *YG101* mutant, the 4.0-kb *Hin*dIII fragment is replaced by a 7-kb *Hin*dIII fragment. The pattern is consistent with integration at the *YG101* site, since the new fragment is 3 kb larger than the wild-type fragment, which is the expected increase in size due to the *LEU2* insertion. In a single *YG103* mutant, the 2.1-kb *Hin*dIII fragment is replaced by two *Hin*dIII fragments of 2.2 and 1.6 kb. This

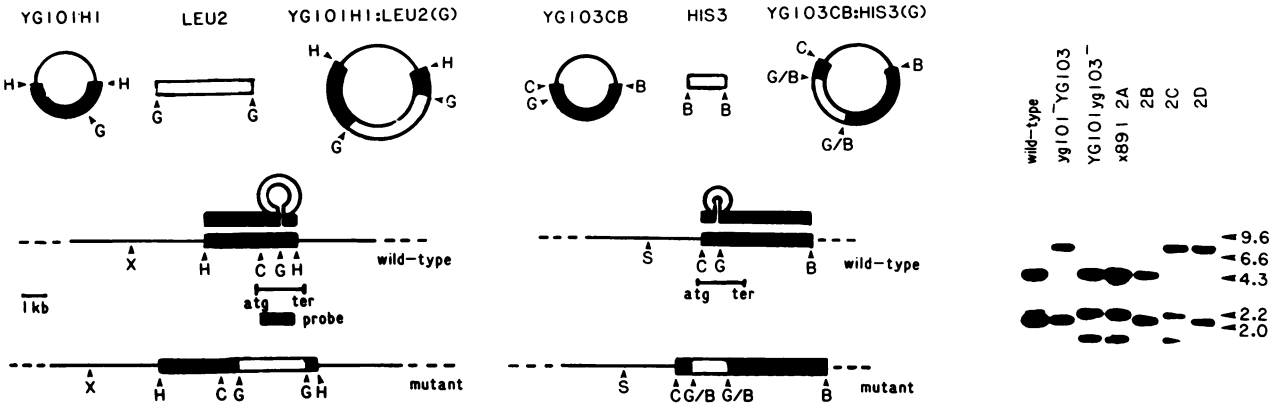


FIG. 2. Schematic drawing of the transformation with *YG101* and *YG103* in vitro constructed mutations and analysis of DNA of resulting transformants. The plasmids YG101H1 and YG103CB were cleaved with the restriction enzyme *Bgl*II, which cleaves at the codon for *aa328*. A 3-kb *Bgl*II fragment isolated from YEp13 (4) carrying the *LEU2* gene, and a 1.7-kb *Bam*HI fragment isolated from YIp1 (34) carrying the *HIS3* gene were ligated to the *YG101*- and *YG103*-containing plasmids, respectively, to form YG101H1:LEU2(G) and YG103CB:HIS3(G). YG101H1:LEU2(G) and YG103CB:HIS3(G) were cleaved with *Hin*dIII and *Cla*I-*Bam*HI, respectively; the linear DNA fragments were used to transform a Leu^- *S. cerevisiae* strain (X46) to Leu^+ and a His^- strain (T88) to His^+, respectively. Several transformants had replaced the wild-type *YG101* or *YG103* region with the gene disruptions as shown. Symbols for restriction endonuclease sites: C, *Cla*I; B, *Bam*HI; X, *Xho*I; H, *Hin*dIII; G, *Bgl*II; S, *Sac*I. G/B indicates that a *Bgl*II end and a *Bam*HI end have been ligated together, resulting in the destruction of both sites. The analysis of DNA of haploids derived from one tetrad is shown in the right-hand panel. The first three lanes are controls: wild type (T88); *yg101*$^-$ *YG103* (T134); *YG101 yg103*$^-$ (T145). The last four lanes contain the DNA from the four haploid strains obtained from a single ascus: 2A, *YG101 yg103*$^-$; 2B, wild type; 2C, *yg101*$^-$ *yg103*$^-$; 2D, *yg101*$^-$ *YG103*.

TABLE 2. *S. cerevisiae* strain doubling times[a]

S. cerevisiae strain designation	Doubling time (h) at a growth temperature of:			
	19°C	23°C	30°C	37°C
YG101 YG103	4.6	3.9	2.0	2.7
yg101⁻ YG103	4.5	3.7	2.1	2.7
YG101 yg103⁻	4.6	4.0	2.0	2.6
yg101⁻ yg103⁻	11	8.6	3.2	2.9
yg101⁻ yg103⁻ + int *YG101*[b]	ND[c]	3.8	2.0	ND
yg101⁻ yg103⁻ + int *YG103*[b]	ND	3.8	1.9	ND

[a] The optical density at 600 nm of cells growing at the indicated temperatures were determined at intervals, and the doubling time was also calculated.

[b] Growth rates are given for X89I.1b (*yg101⁻ yg103⁻*) containing an integrated (int) wild-type copy of *YG101* or *YG103* as described in the text.

[c] ND, Not determined.

pattern is consistent with integration at the *YG103* site, since the *HIS3* gene has an internal *Hin*dIII site resulting in two fragments that hybridize to the probe.

The recovery of Leu$^+$ and His$^+$ segregants from the diploids indicates that the *YG101* and *YG103* mutations do not result in cell inviability. No altered phenotype of the mutant haploid strains has been observed. These strains grow at rates very similar to those of wild-type strains at several temperatures (Table 2). Our results suggest that neither *YG101* nor *YG103* separately has an essential function, since no phenotypic change is associated with haploid strains lacking either an intact *YG101* or *YG103* gene. It is possible that these genes have overlapping functions. To test this possibility, we constructed a diploid (X89) that is heterozygous for mutant and wild-type *YG101* and *YG103*, by mating a *yg101⁻ YG103* and a *YG101 yg103⁻* strain and then sporulating it to obtain haploid segregants. Overall, the segregation of the His$^+$ and Leu$^+$ markers was as expected (parental ditype, nonparental ditype, and tetratype asci in the ratio of 1:1:4) if the loci for *YG101* and *YG103* were unlinked. The Leu$^+$ His$^+$ haploids ought to contain both mutant *YG101* and *YG103* genes, and no corresponding wild-type genes. This prediction was verified by hybridization analysis of four haploid segregants of a tetrad (Fig. 2).

Initially, it was observed that the Leu$^+$ His$^+$ cells grew more slowly on the dissection plates. The altered growth pattern of the double mutant was studied at several temperatures. Differences in growth rates in liquid medium between the wild-type and double mutant were found at all temperatures, but the disparity was greatest at lower temperatures (Table 2, Fig. 3). At 37°C the double mutant grew only 1.1 times slower than the wild type, a doubling time of 2.9 h compared to 2.7 h. As the temperature was lowered, the differential between the strains became larger. The double mutant grew 1.6 times slower at 30°C, 1.9 times slower at 23°C, and 2.4 times slower at 19°C (Fig. 3). Wild-type *S. cerevisiae* grows optimally at 30°C and about 35% more slowly at 37°C, whereas *yg101⁻ yg103⁻* mutants grow slightly better at 37°C than at 30°C.

If the slow-growth phenotype observed was due to the absence of both *YG101* and *YG103* functional gene products, reintroduction of either the *YG101* or the *YG103* wild-type gene should reverse the phenotype. We reintroduced each of the wild-type genes into the double-mutation strain by integration into the chromosome. Wild-type *YG101* was reinserted by integration of plasmid B2, which contains the entire *YG101* gene as well as the *URA3* gene. Integration was directed to the *YG101* locus (27) by linearizing the plasmid by digestion with *Xho*I, which cleaves in the *YG101* flanking region (Fig. 2). Similarly, the wild-type *YG103* gene was reintroduced into the double mutant by integration of the plasmid E3 which contains the entire *YG103* gene and the *URA3* gene. Before transformation this plasmid was linearized by digestion with *Sac*I, which cleaves in the *YG103* flanking sequences. The growth rates of strains containing the integrated *YG101* or the *YG103* genes, as well as the mutant *YG101* and *YG103* genes were determined. Cells containing either the reintroduced *YG101* or *YG103* gene grew at the rate of the wild-type strains (Table 1). We conclude that the slow growth of the *yg101⁻ yg103⁻* strains is due to the absence of a wild-type *YG101* gene and a *YG103* gene and that this slow-growth phenotype can be complemented by either *YG101* or *YG103*.

The proteins synthesized in wild-type and mutant strains were analyzed to attempt to identify the proteins encoded by *YG101* and *YG103*. Cells were labeled with [^{35}S]methionine during logarithmic growth at 23°C, and the proteins were displayed on two-dimensional gels (Fig. 4). No differences in the pattern of spots were observed between the wild-type strain and the two single-mutation strains. However, an intense spot and an adjacent, less intense spot, representing proteins of approximately 67 kilodaltons, were absent from the double mutant. The size of the protein encoded by the *YG101* gene as predicted from analysis of the DNA sequence is 66,596 daltons (L. Stinson, M. Slater, and E. Craig, unpublished data). The synthesis of the proteins absent in the double mutant is dramatically decreased upon temperature upshift (Fig. 4). Therefore, the size of the protein and the regulation of expression upon temperature upshift is consistent with the notion that the protein spots, absent in the double mutant, are encoded by *YG101* and *YG103*. Since both spots are present in the single mutants, it is likely that the additional spot is the result of a modification causing a single charge alteration, which occurs on both the *YG101*- and *YG103*-encoded proteins.

Analysis of promoter-structural gene fusions. Although the

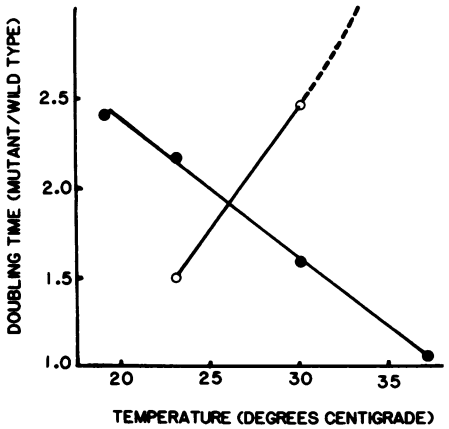


FIG. 3. The effect of absence of the *YG100/YG102* and *YG101/YG103* gene pairs on growth rate at different temperatures. The ratios of the doubling time of mutant cells to wild-type cells and the indicated temperatures were plotted. A ratio of 1.0 indicates the mutant strain is growing with the same doubling time as the wild-type strain. The larger the ratio, the greater the difference between the doubling times. The dashed line at higher temperatures indicates that *yg100⁻ yg102⁻* cells do not grow at 37°C. Strain designations: *yg100⁻ yg102⁻* (○); *yg101⁻ yg103⁻* (●).

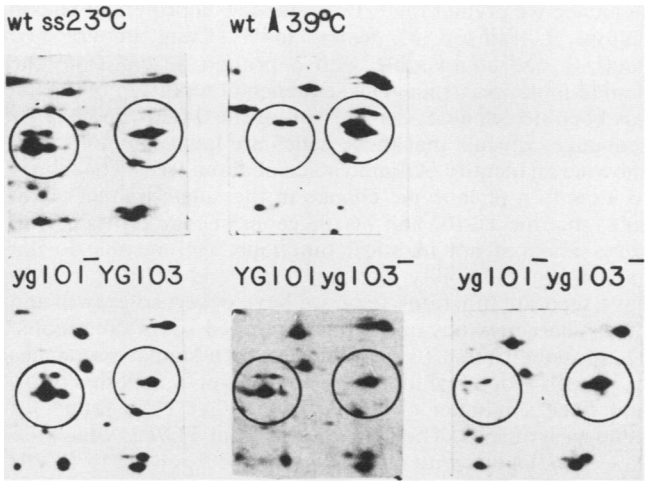


FIG. 4. Proteins synthesized in wild-type and mutant strains. Cells grown in supplemented minimal media lacking methionine and uracil at 23°C were labeled with [^{35}S]methionine for 15 min. Cells were labeled either during growth at 23°C or 15 min after shift to 39°C (upper right panel only). The labeled proteins were analyzed by two-dimensional gel electrophoresis as described in the text. Fluorographs of a portion of the gels which include *hsp70*-related proteins are shown. The circles encompass spots shown by analysis of mutant strains and antibody reactivity to be *hsp70*-related proteins (K. Palter, K. Jacobsen, and E. A. Craig, unpublished observations).

phenotypes of the *yg101*$^-$ *yg103*$^-$ and the *yg100*$^-$ *yg102*$^-$ strains (6) are different, the question arises as to whether the protein products encoded by these related genes perform distinctly different functions. Since the genes are expressed differently at disparate temperatures, it is possible that the proteins would be able to compensate for each other if present in the appropriate amounts. To attempt to test this idea, fusions were constructed which put both the *YG101* structural gene under the control of the *YG102* promoter and the *YG100* structural gene under the control of the *YG101* promoter. As described in Materials and Methods, the promoters and structural genes were fused in the 5′ transcribed untranslated region. The fusions were inserted into the centromere containing vector YCp50 and transformed into wild-type and double-mutation strains. To test whether the proteins expressed from the fusions were regulated as expected from the adjacent promoter, proteins synthesized in the double-mutation strains containing the fusions were analyzed (data not shown). As expected, in the *yg101*$^-$ *yg103*$^-$ strain containing the *YG102* promoter-*YG101* structural gene fusion, the *YG101-YG103* spot which was absent in the double mutant is now present, but its synthesis is no longer repressed upon a heat shock. Similarly, the *yg100*$^-$ *yg102*$^-$ strain containing the *YG101* promoter-*YG100* structural gene fusion has a spot previously identified as the *YG100* gene product. However, instead of being enhanced upon heat shock, its synthetic rate is diminished. Therefore, we conclude that the fusion genes are being regulated as expected.

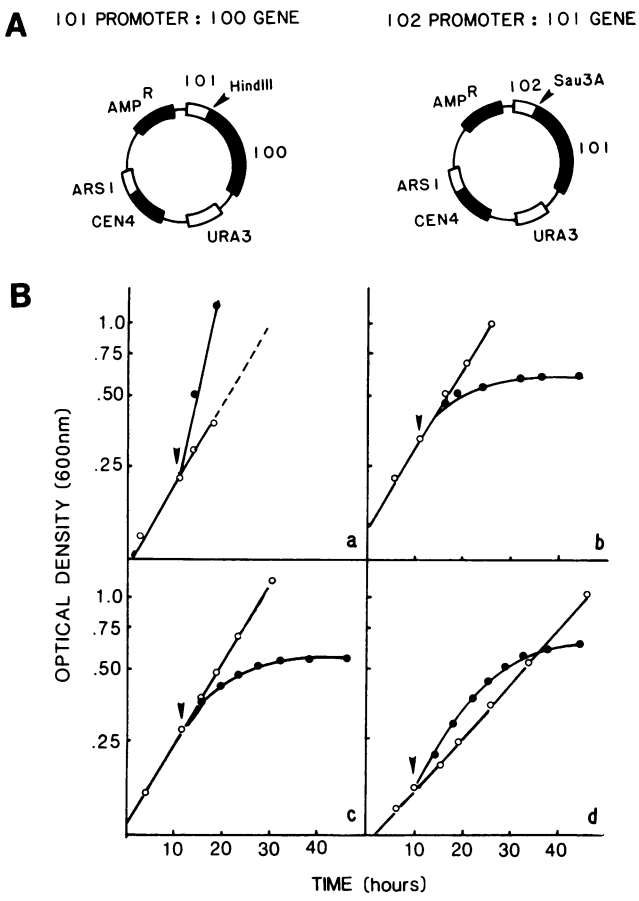
As reported in Fig. 5, the doubling times at 23°C of the wild-type and the *yg101*$^-$ *yg103*$^-$ strains in the presence and absence of the fusions were determined. The doubling time of the wild-type strain was 4.2 h; the *yg101*$^-$ *yg103*$^-$ strain in the absence of a fusion vector doubled in 10 h. In the presence of the *YG101* promoter-*YG100* structural gene fusion the growth rate was unchanged, with a doubling time of 10.3 h. However, in the presence of the *YG102* promoter-*YG101* structural gene fusion a doubling time very similar to that of the wild type (4.1 h) was attained. Therefore, the *YG100* gene product is unable to rescue the relative cold sensitivity of the *yg101*$^-$ *yg103*$^-$ strain even when its expression is under the control of the *YG101* promoter. However, *YG101* protein, even under the control of the *YG102* promoter, can rescue the slow-growth phenotype.

The growth of the *yg100*$^-$ *yg102*$^-$ mutant in the presence and absence of the fusions was analyzed after an upshift from 23 to 37°C, conditions that most dramatically demonstrate the phenotypic effect of the absence of the *YG100*- and *YG102*-encoded proteins. Wild-type cells increase their growth rate upon an upshift (Fig. 5). When the double mutant, growing logarithmically at 23°C, was transferred to 37°C, no immediate lag in growth was observed. However, after several hours there was no further increase in optical density. A very similar inhibition in growth at 37°C occurred in the *YG100-YG102* double mutant containing the *YG102* promoter-*YG101* structural gene fusion. Growth of the double mutant containing the *YG101* promoter-*YG100* structural gene fusion was also inhibited at 37°C; however, it grew slightly better at 37°C than the strain lacking the fusion. This double mutant is able to undergo two divisions, compared to less than one division for the double mutant with or without the *YG102* promoter-*YG101* gene fusion. It is likely that the *YG101* promoter-*YG100* structural gene fusion cannot fully rescue the temperature-sensitive phenotype of the *yg100*$^-$ *yg102*$^-$ strain because of decreased transcription from the *YG101* promoter upon temperature upshift.

DISCUSSION

The data presented here represent a genetic test of the in vivo role of two cognate genes coding for products related to the heat-inducible 70-kilodalton heat shock protein. By in vitro mutagenesis and transformation, two yeast cognate genes, *YG101* and *YG103*, were replaced by insertion mutations. Analysis of single-mutation strains revealed no altered phenotype at any stage of the life cycle or at any growth temperature. However, haploid strains containing mutations in both *YG101* and *YG103* had altered growth properties. Whereas wild-type yeast strains have an optimal growth temperature of 30°C, the growth rate of the double-mutation strains was faster at 37°C than at 30°C. At 37°C the double mutant grew nearly as well as the wild-type strain (a doubling time of 2.9 h compared to 2.7 h for wild type). However, as the temperature was lowered, the difference in growth rates became greater. These results suggest that the cognate proteins provide a function needed for normal logarithmic growth at all temperatures; however, the lower the temperature the greater the effect of the absence of these cognate proteins.

The reduced growth rates of strains with mutations in both the *YG101* and *YG103* genes at all temperatures indicate that their protein products are important for normal growth under a variety of conditions. This is not surprising since RNA encoded by *YG101* or *YG103*, or both, is present at all of the temperatures examined. The fact that the absence of these proteins affects growth more at lower than at higher temperatures is consistent with the fact that *YG101* and *YG103* transcripts are of higher abundance during steady-state growth at lower temperatures. Therefore, the severity of the observed phenotype varies in parallel with the variation in the level of expression of the genes. *YG101*- and *YG103*-encoded proteins are among a small class of proteins normally expressed during steady-state growth at 23°C whose



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STRAIN	FUSION	DOUBLING TIME (HOURS) 23°C
WT	--	4.2
YG101-YG103-	--	10
YG101-YG103-	101p-102SG	10.3
YG101-YG103-	102p-101SG	4.1

FIG. 5. Growth of strains containing promoter-structural gene fusions. (A) Fusions of *YG102* promoter-*YG101* structural gene and *YG101* promoter-*YG100* gene in the centromere containing vector YCp50 (15). (B) Cultures of wild type (T18) (panel a), *yg100*$^-$ *yg102*$^-$ (T128) (panel b), T128 with the *YG102* promoter-*YG101* gene fusion (panel c), and T128 with the *YG101* promoter-*YG100* gene fusion (panel d) were grown at 23°C and then shifted to 37°C at the times indicated by the arrow. Symbols: ○, 23°C; ●, 37°C. (C) The optical density at 600 nm of cells growing at 23°C were determined at intervals, and the doubling time was also determined. The wild-type and *yg101*$^-$ *yg103*$^-$ strains used were T128 and X89 1B.

synthesis is decreased following a temperature upshift (22, 24). The reduction in the synthesis of *YG101*- and *YG103*-encoded proteins can be accounted for solely by the drop in RNA levels; translational control mechanisms need not be involved. Unlike *D. melanogaster* (2, 23), it is likely that yeasts do not exert translational control to reduce expression of normal proteins, but rather the reduction is accomplished mainly through transcriptional inhibition and degradation of preexisting RNA as reported previously by Lindquist (20).

The entire sequence of one of the two cognate genes, *YG101*, has been determined. From analysis of the DNA sequence we predict that *YG101* encodes a protein of 66,596 daltons (L. Stinson, M. Slater, and E. Craig, unpublished data), a size comparable with a protein absent from the double-mutation strain. The sequence of a portion of *YG103* has been determined; comparison of the *YG101* and *YG103* sequences reveals that these genes are highly homologous, showing an identity of amino acids of about 94%. The failure to identify a phenotypic change in the single mutants indicates that the *YG101* and *YG103* genes encode proteins with very similar if not identical functions. Our results do not exclude the possibility that the *YG101* and *YG103* products have separate functions since we have observed growth and other characteristics under only a limited set of conditions. The possibility that two proteins of such similar sequences have nearly identical functions is not surprising both a priori and since a number of similar cases have been found for other yeast genes. The genes *YG100* and *YG102*, which are 96% homologous with each other and 65% related to *YG101* and *YG103*, seem to have nearly identical functions, since disruption of only one of the gene pair causes no obvious phenotypic change (6). Similar results have been obtained with pairs of genes encoding histones H2A and H2B (29) and ribosomal protein p51 (1), as well as the *ras*-related proteins (16, 35) of *S. cerevisiae*.

The *hsp70* gene family of *S. cerevisiae* offers an opportunity to study a number of related proteins in a simple unicellular organism. The yeast genome contains at least eight genes in the *hsp70* multigene family (7). Mutational analysis has been carried out on two highly homologous pairs of genes, the *YG101*/*YG103* pair and the *YG100*/*YG102* pair. As described previously (6), strains lacking both a functional *YG100* gene and a *YG102* gene showed altered growth properties at all temperatures, but the higher the temperature the more dramatic the effect of the absence of these genes. At 37°C *yg100*$^-$ *yg102*$^-$ cells are unable to form colonies. As reported here, strains lacking both a functional *YG101* gene and a *YG103* gene show altered growth properties, particularly at lower temperatures. This difference in phenotype caused by mutations in related genes of the multigene family could represent differences in the function of related proteins. On the other hand, the different phenotypes could be due to the absence in the double-mutation strains of sufficient quantities of related proteins with very similar functions to maintain wild-type growth rates. A test to distinguish these two possibilities was carried out by putting the expression of *YG100* and *YG101* under the control of the *YG101* and *YG102* promoters, respectively. The inability of the related proteins, even under the control of the appropriate promoter, to rescue the mutant phenotypes leads us to conclude that the products of the *YG101*/*YG103* and *YG100*/*YG102* gene pairs perform significantly different functions and cannot compensate for one another. Since the functions of all the *hsp70*-related proteins are unknown, it is difficult to assess how different the function of related proteins might be. It is possible that similar functions are performed, but the temperature optima for the proteins are very different.

Multigene families are prevalent in eucaryotic organisms. In most cases, the capacity of related proteins to carry out the same functions has not been determined. In a few cases it has been shown that the related genes can compensate for the mutated gene if expressed in appropriate amounts, although it is likely that the related proteins have slightly different roles under different physiological conditions. For example, *iso*-1-cytochrome *c* (*CYC1*) and *iso*-2-cytochrome *c* (*CYC7*), which are 83% identical in amino acid sequence

(25), normally constitute, respectively, 95 and 5% of the total cytochrome *c* complement in *S. cerevisiae* (32). *cyc1*$^-$ *CYC7*$^+$ cells will not grow on lactate medium (32); revertants that can grow on lactate show an increase in synthesis of the wild-type *CYC7* gene (19). Therefore, the proteins encoded at the *CYC1* and *CYC7* loci are able to compensate for one another. This example is similar to the cases of the *YG101/YG103* and *YG100/YG102* gene pairs in the sense that the presence of the protein encoded by only one of each of the gene pairs need be present for normal growth.

In complex organisms, proteins encoded by multigene families are often expressed in different cell types. For example, some of the multiple actin (10) and tubulins (18) of *D. melanogaster* are expressed in only a limited number of tissues during different developmental stages. Whether some of these related genes perform compensatable functions in the different tissues is an open question, and the answer awaits the expression of related proteins in tissues in which they are not normally present. Tissue-specific expression of members of a multigene family need not imply functional differences in the proteins. For example, analysis of strains containing mutations in the testes-specific tubulin (17) indicates that the β-2 tubulin subunit that forms the sperm axoneme is involved in multiple functions during spermatogenesis, including the formation of the meiotic spindle and cytoplasmic microtubules. The suspected involvement of the testes-specific tubulin in structures found in all cells suggests that this tissue-specific tubulin might be capable of functioning in other cell types as well. Therefore, it is not clear how commonly the inability of related proteins to compensate for one another, as is the case with the *YG101/YG103* and *YG100/YG102* gene pairs, will be found.

Heat shock or related proteins likely function during normal growth in a variety of species, since there are a number of reports of expression of these proteins in nonstressful situations. For example, DnaK, which is related to Hsp70 of eucaryotes, is an abundant protein at all growth temperatures (12). Cognate proteins, related to Hsp70, are abundant throughout development in *D. melanogaster*. The induction of heat shock proteins by a wide variety of stresses suggests that these proteins function to allow growth and survival under a diversity of conditions. One stress is subjection to cold temperatures. The data reported here are the first indication that proteins related to the heat shock proteins may function to allow cells to grow better at temperatures below the optima.

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